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EFFECT OF MYOPLASMIC pH ON EXCITATION–CONTRACTION COUPLING IN SKELETAL MUSCLE FIBRES OF THE TOAD

BY G. D. LAMB, E. RECUPERO AND D. G. STEPHENSON

From the Department of Zoology, La Trobe University, Bundoora, Melbourne, 3083, Australia

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SUMMARY

1. The effect of pH on excitation–contraction coupling in skeletal muscle of the toad was examined using a skinned fibre preparation which gives ready access to the intracellular environment while still allowing stimulation of Ca^{2+} release by the normal voltage-sensor mechanism.

2. In each fibre, depolarization-induced responses (produced by changing the ions in the bathing solution) were examined first at pH 7·1, and then at another pH between 6·1 and 8·0. At all pH levels examined, the first depolarization elicited a large response which was slightly greater (pH 7·6 and 8·0) or smaller (pH 6·6 and 6·1) than that at pH 7·1. The size of the first depolarization-induced response varied with pH in almost exactly the same manner as did the maximum Ca^{2+}-activated response. The duration of the depolarization-induced response at all other pH levels was longer than at pH 7·1.

3. Repeated depolarizations (30 s or more apart) produced similar responses at pH 7·1, but at all other pH levels examined the second and subsequent responses became progressively smaller. The reasons for this decrease were different at low and high pH.

4. Examination of the size of the depolarization-induced response after reloading the depleted sarcoplasmic reticulum (SR) with Ca^{2+} in solutions at various pH levels, indicated that the SR Ca^{2+} pump operated more poorly at pH 6·6 and 6·1 than at pH 7·1. This can account for the wide and successively smaller depolarization-induced responses at acidic pH.

5. Examination of the size of the depolarization-induced response after 5 min bathing without stimulation in a very low $[Ca^{2+}]$ solution ($-\log_{10}[Ca^{2+}]$ (pCa) > 8·0, 1 mM-BAPTA (1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), indicated that the sarcoplasmic reticulum (SR) lost far more Ca^{2+} at pH 7·6 and 8·0 than at pH 7·1 or 6·1. This indicates that SR is 'leakier' at alkaline pH.

6. Exposure of fibres heavily loaded with Ca^{2+} to solutions at pH 8·0 invariably induced a large response within 1–6 s. This response was unaffected by inactivation of the voltage sensors and was blocked completely by 2 μM-Ruthenium Red. This response and the 'leak' at alkaline pH are consistent with previous studies of the pH dependence of Ca^{2+}-activated opening of single Ca^{2+} release channels in lipid bilayers.

7. These findings indicate that depolarization can induce massive Ca^{2+} release at

both acidic and alkaline pH, provided that the SR is normally loaded with Ca^{2+}, and give further insight into the intracellular events underlying fatigue in skeletal muscle.

INTRODUCTION

Intracellular pH affects many different functions in skeletal muscle fibres, such as the Ca^{2+} activation of the contractile proteins (Ashley & Moisescu, 1977; Fabiato & Fabiato, 1978) and the functioning of the Ca^{2+} pump in the sarcoplasmic reticulum (SR; MacLennan, 1970). Recently, it has been shown that lowering the 'myoplasmic' pH to 6·5 almost completely prevents the Ca^{2+}-activated opening of single SR Ca^{2+} release channels in lipid bilayers, in the absence of ATP and Mg (Ma, Fill, Knudson, Campbell & Coronado, 1988; Rousseau & Pinkos, 1990). This could imply that normal excitation–contraction (E–C) coupling is greatly hampered at such intracellular pH and that this at least partly accounts for the decrease in force observed in fatigued muscle. However, even though such single-channel studies provide valuable information on channel function, one cannot directly extrapolate these findings to normal E–C coupling (Endo, 1985; Lamb & Stephenson, 1990*b*, 1991). These findings with single release channels are not easily reconciled with the fact that intracellular acidification of intact fibres with CO_2 only caused a small reduction in maximum tetanic tension (Curtin, 1986; Renaud, Allard & Mainwood, 1986; Lännergren & Westerblad, 1991), and actually produced an increase in the aequorin light signal (Allen, Lee & Westerblad, 1989).

Consequently, we have examined the effect of intracellular pH on E–C coupling in a preparation in which Ca^{2+} release is controlled by the normal voltage-sensor mechanism and in which factors such as the level of Ca^{2+} loading in the SR, are under experimental control (Lamb & Stephenson, 1990*a*). The effect of pH on the 'leak' of Ca^{2+} from SR and the activity of the SR Ca^{2+} pump, were also examined in this same preparation, thereby giving a more complete view of the movement of Ca^{2+} at various pH levels. These data provide important information about one of the factors possibly influencing fatigue of skeletal muscle.

METHODS

The skinned fibre preparation described previously (Lamb & Stephenson, 1990*a*) was used, with the modifications to the bathing solutions described below. Briefly, cane toads (*Bufo marinus*) were stunned by a blow to the head and then double pithed and the iliofibularis muscles were removed. The excised muscles were pinned out under paraffin oil and a segment of a single fibre was mechanically skinned and attached to a force transducer (AME875) to measure isometric contractions, which were recorded on a chart-recorder (Linear, Reno, USA). The resting length (L) and diameter (D) of the fibre were measured in paraffin oil and then the fibre was stretched a further 20% (final sarcomere length 2·6–2·8 μm). The skinned fibre was then placed in a 2 ml Perspex bath filled with K–HDTA solution (HDTA, hexamethylenediaminetetraacetate) with 1 mM-free Mg^{2+} for 2 min before being stimulated by rapidly removing the bath and replacing it with another filled with an appropriate solution.

BAPTA (1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) was used to buffer Ca^{2+} in most experiments, instead of EGTA (ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), because the affinity of BAPTA for Ca^{2+} is not strongly affected by pH (estimated apparent affinity constant for the ionic strength, temperature and $[Mg^{2+}]$ used here: 1·7, 5·5 and 6·7 × 10^6 M^{-1} at pH 6·1, 7·1 and 8·0 respectively (Harrison & Bers, 1987)). The BAPTA (Molecular Probes, OR,

USA) was titrated against Ca^{2+} and found to be 72% pure; concentrations of BAPTA used here have been corrected accordingly. The standard K–HDTA solution contained (mM): K^+, 117; Na^+, 36; $HDTA^{2-}$, 50; total ATP, 8·0; total Mg, 8·5; creatine phosphate, 10; total BAPTA or EGTA, 0·05; HEPES buffer, 60; NaN_3, 1; pCa 6·7; with added KOH to pH 7·10±0·01 at 23 °C. The resulting free $[Mg^{2+}]$ was 1 mM and the osmolality was 255 mosmol/kg. (The azide (NaN_3) was present in order to prevent the mitochondria from interfering with the calcium movements.) The Na–HDTA solution had the same composition as the potassium solution except that all K^+ (117 mM) was replaced by Na^+. The choline chloride (ChCl) solution contained (mM): ChCl, 103; Na^+, 42; total Mg, 8·15; total ATP, 8; creatine phosphate, 10; HEPES buffer, 20; EGTA or BAPTA (total), 0·05; pCa 6·7 and pH 7·10±0·01. The osmolality was close to 255 mosmol/kg.

Corresponding K– and Na–HDTA and ChCl solutions at different pH were made with 50 μM-BAPTA. The osmolality was kept constant by altering the amount of HEPES (and ChCl where appropriate) and for better buffering at acid pH PIPES (piperazine-*N*-*N*′-bis(2-ethane sulphonic acid)) was also added. The [HEPES] was 45, 50, 50 and 44 mM in the potassium and sodium solutions and 10, 20, 20 and 35 mM in the ChCl solutions, at pH 6·1, 6·6, 7·6 and 8·0 respectively. The pH 6·1 and 6·6 solutions had additionally 13 and 6·6 mM-PIPES, respectively. The [ChCl] was reduced from 100 to 80 mM in the pH 8·0 ChCl solution. The free $[Mg^{2+}]$ was kept at 1 mM by adjusting the total Mg added according to the expected changes in the apparent affinity constants (see Sillén & Martel, 1970; Ashley & Moisescu, 1977): 6·65, 7·8, 9·25 and 11·1 mM-total Mg in the HDTA solutions and 6·65, 7·6, 8·25 and 8·4 mM-total Mg in the ChCl solutions at pH 6·1, 6·6, 7·6 and 8·0 respectively.

The maximum-activating solutions (pCa 4·0) were similar to the corresponding K–HDTA solutions but contained 50 mM-total EGTA instead of HDTA and the total Mg was adjusted to give 1 mM-free Mg^{2+} (Ashley & Moisescu, 1977; Stephenson & Williams, 1981). The 'heavily buffered' solutions used to load Ca^{2+} at different pH (Figs 6 and 7) all had 1 mM-Ca-BAPTA, and were made from the appropriate K–HDTA solution by adding Ca-HDTA and BAPTA (from stock solutions of the same ionic strength) to give total [BAPTA] of 1·61, 1·27 and 1·18 mM at pH 6·1, 6·6 and 7·1 respectively (pCa 6·0 solutions) and 3·67 and 2·82 mM at pH 6·6 and 7·1 respectively (pCa 7·0 solutions). In other experiments where it was desirable to load the preparation quickly at pH 7·1 without having to subsequently wash out the high [BAPTA], a 'weakly buffered' Ca^{2+}-loading solution (pCa 5·7, 50 μM-EGTA) was used; this solution was made from the standard K^+ solution by adding $CaCl_2$.

In all figures, the fibre was bathed in K–HDTA solution unless indicated otherwise and the pH of any bathing solution was 7·1 unless indicated otherwise. Data are expressed as means(±S.E.M) throughout. All experiments were performed at room temperature (23±2 °C).

RESULTS

Ca^{2+}-activated responses

The effect of pH on Ca^{2+}-activated force has been studied previously in the frog (Fabiato & Fabiato, 1978; Robertson & Kerrick, 1979) and was examined here to make accurate comparison with the depolarization-induced responses obtained in the same preparation and under the same conditions (i.e. osmolality, free $[Mg^{2+}]$, [Mg-ATP] etc). The maximum Ca^{2+}-activated force was decreased at acid pH and increased at alkaline pH (see Fig. 3), with the changes being slightly smaller than those found previously in the frog. The pCa (i.e. $-\log_{10}[Ca^{2+}]$) producing half-maximum force was approximately 6·25, 6·15, 5·9, 5·65 and 5·25 in pH 8·0, 7·6, 7·1, 6·6 and 6·0 respectively (mean data from at least four fibres at each pH). The value for pH 7·1 lies between the two values reported previously for pH 7·0 in the frog (Fabiato & Fabiato, 1978; Robertson & Kerrick, 1979) and the relative shift with pH is very similar to both those studies. Thus, the fibre became progressively less sensitive to Ca^{2+} at lower pH. As reported previously (Robertson & Kerrick, 1979), it was difficult to examine the Ca^{2+}-activated responses at pH 8·0, because the force

deteriorated rapidly and permanently. However, this was not a problem in low [Ca^{2+}] at pH 8·0 (see below).

Depolarization-induced responses

Depolarization-induced responses were studied in solutions very weakly buffered for Ca^{2+} (50 μM-total EGTA or BAPTA). At pH 7·1, large depolarization-induced responses could be elicited in virtually every fibre by substituting all the K^+ in the standard K–HDTA solution with Na^+ or all the K–HDTA with ChCl, as described previously (Lamb & Stephenson, 1990*a*). In experiments described below, qualitatively similar results were obtained with either type of depolarizing stimulus, though the values documented were obtained with ChCl stimuli to ensure that the fibre was depolarized maximally in each case. (Fibres used here were not subjected to ChCl substitution when heavily loaded with Ca^{2+}, thus avoiding any contribution from the abnormal Ca^{2+} release which can occur with ChCl substitution (see Lamb & Stephenson, 1990*a*).) At pH 7·1, the mean depolarization-induced response (ChCl substitution) was 78% ($\pm$3%, $n = 34$) of the maximum Ca^{2+}-activated force in the same fibre. There was no noticeable difference in the depolarization-induced responses obtained in the same fibre when either 50 μM-EGTA or 50 μM-BAPTA was used to buffer Ca^{2+} (pCa $\approx$ 6·7) in both the polarizing and depolarizing solutions. As described previously (Lamb & Stephenson, 1990*a*), successive depolarizations, at least 30 s apart, elicited similar responses, until the fibre eventually 'ran down' after approximately ten to twenty-five responses.

In all the following experiments each fibre was first subjected to two or three depolarizations under the standard conditions at pH 7·1, without exposure to any of the 'loading solutions' (see Methods), in order to verify that E–C coupling was functional and the responses were reproducible and to establish a measure with which to compare subsequent responses.

Depolarization-induced responses at different pH

Acidic pH

The first depolarization after 1 min equilibration in acidic pH (either 6·6 or 6·1) invariably induced a large response, which was slightly smaller than the preceding response at pH 7·1 (e.g. Fig. 1). In every fibre, the relative size of the first depolarization-induced response at acidic pH (compared to that at pH 7·1) was very similar to the relative size of the maximum Ca^{2+}-activated force at that pH (Fig. 3). The duration of the depolarization-induced response at acidic pH was longer than at pH 7·1; 2·18 ($\pm$0·13) times longer at pH 6·1 (nine fibres) and 1·37 ($\pm$0·05) times longer at pH 6·6 (five fibres), measured at half-maximum. At both pH 6·1 and 6·6, the response to the second depolarization was markedly smaller than to the first depolarization (Figs 1 and 3). After returning to pH 7·1 for 30 s, the depolarization-induced response was further reduced (mean response reduced by a further 21% and 20% of the control response at pH 7·1, after pH 6·1 and 6·6, respectively). The response to successive depolarizations only increased slightly (6$\pm$2% of the control response, $n = 14$) when a fibre was left in the standard K^+ solution (pH 7·1, pCa 6·7, 50 μM-BAPTA) for 30 s, but fully recovered to control levels if exposed to the weakly buffered Ca^{2+}-loading solution (pH 7·1, pCa 5·7, 50 μM-BAPTA) for 30–60 s before

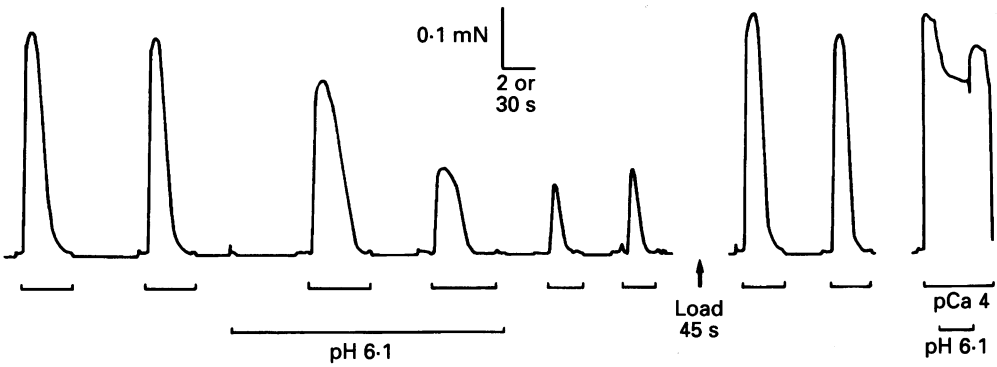

Fig. 1. Effect of low pH on successive depolarization-induced responses. The first depolarization-induced response at pH 6·1 was reduced relative to the response at pH 7·1 by a similar amount as the Ca^{2+}-activated force (pCa 4·0) (extreme right). The reduced depolarization-induced response upon returning to pH 7·1 and its subsequent recovery upon Ca^{2+} loading, indicate that the SR was depleted by the depolarizations in pH 6·1. The unlabelled bars indicate depolarization by ChCl substitution. Time scale: 2 s during depolarizations and 30 s elsewhere. Fibre loaded in weakly buffered load solution (50 μM-EGTA, pCa 5·7) at arrow. *L*, 1·6 mm; *D*, 38 μm.

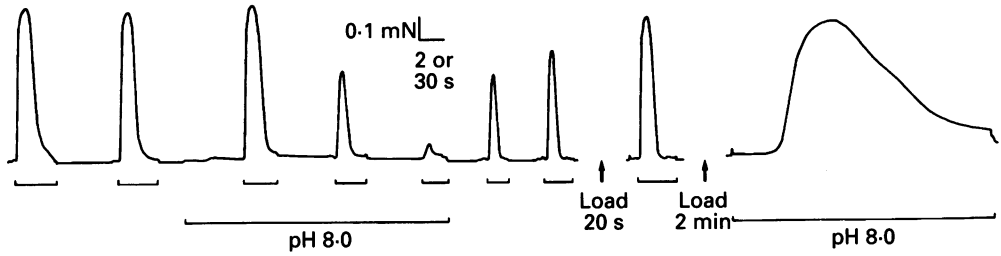

Fig. 2. Effect of high pH on successive depolarization-induced responses. The unlabelled bars indicate depolarization by ChCl substitution. Note that the first depolarization-induced response after returning from pH 8·0 to 7·1 is reduced relative to the initial response (indicating some depletion), but is not as small as the preceding response in pH 8·0 (see text). After heavily loading the SR with Ca^{2+} (2 min in weakly buffered load solution), exposure to pH 8·0 (K^+) solution induced a large response without any depolarization (extreme right). Time scale: 2 s during depolarization and alkaline pH-induced response (extreme right) and 30 s between depolarizations. *L*, 2·3 mm; *D*, 53 μm.

being returned to the standard solution and depolarized (e.g. Fig. 1). This clearly shows that the SR had been depleted of Ca^{2+} by the bathing and depolarizations in acidic pH. This was further confirmed by the substantially reduced response to caffeine (1 or 30 mM) observed in experiments with several other fibres after depolarizations in acidic pH (not shown). In other experiments, EGTA was used instead of BAPTA to buffer Ca^{2+} in both the polarizing and depolarizing solutions, and the relative response to the first and second depolarizations in acidic pH were similar to that found with BAPTA (70 and 38% at pH 6·0; 100 and 93% at pH 6·6, $n = 4$), despite the relatively high [EGTA] necessary to adequately buffer the [Ca^{2+}] around pCa 6·7 at acidic pH (1·2 and 0·3 mM in pH 6·0 and 6·6 respectively).

Alkaline pH

When depolarized in alkaline conditions (pH 7·6 or 8·0), the first depolarization-induced response was usually slightly larger than the response at pH 7·1 (Fig. 2). The mean relative depolarization-induced response at pH 7·6 was very similar to the

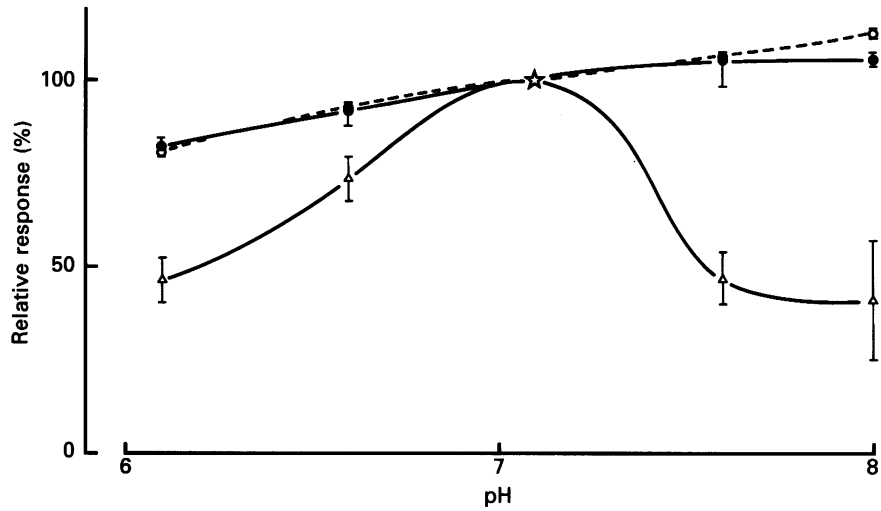

Fig. 3. Mean (±S.E.M.) depolarization-induced responses (●, first depolarization; △, second depolarization) and maximum Ca^{2+}-activated responses (○ with dashed line) at different pH. All responses are expressed as a percentage of the corresponding response at pH 7·1 in the same fibre, ☆ (e.g. see Figs 1 and 2). The fitted curves are arbitrary. Number of fibres: 9, 5, 5 and 6 at pH 6·1, 6·6, 7·6 and 8·0, respectively. Note the close correspondence between the first depolarization-induced response and the maximum Ca^{2+}-activated force.

mean relative Ca^{2+}-activated response, but at pH 8·0 the depolarization-induced response was not increased by as much as the Ca^{2+}-activated force (Fig. 3). As with the responses at acidic pH, the first depolarization-induced response lasted longer at alkaline pH than at pH 7·1 (by a factor of 1·23±0·19 ($n = 5$) and 1·66±0·21 ($n = 6$) at pH 7·6 and 8·0, respectively), and the second depolarization-induced response was much smaller than the first (Figs 2 and 3). Also, as at acidic pH, the response after returning to pH 7·1 for 30 s was substantially lower than the initial control response (39 and 31% of the control response after pH 7·6 and 8·0, respectively), but fully recovered to control levels after brief loading with Ca^{2+} (e.g. Fig. 2). Thus, this implies that the SR became partly depleted of Ca^{2+} by the bathing and depolarizations in alkaline pH. However, in contrast to results in acidic pH, the first depolarization-induced response after returning to pH 7·1 was actually substantially larger than the preceding response in alkaline pH (means increased by 14 and 28% of the control response at pH 7·1, after pH 7·6 and 8·0 respectively) (e.g. Fig. 2). This has interesting implications concerning Ca^{2+} release from the SR at alkaline pH (see Discussion).

Alkaline pH-induced responses

Exposure of heavily loaded fibres to the K^+ solution at pH 8·0 invariably induced a large response (mean 85 ± 6% of the maximum Ca^{2+}-activated force at that pH, $n = 6$) within 1–6 s (mean, 3 ± 1 s, $n = 6$) (e.g. Fig. 2). No such alkaline pH-induced response was observed in fibres that had not been heavily loaded with Ca^{2+}, that is, when they had not been exposed to one of the Ca^{2+}-loading solutions and only had the level of loading present upon skinning and mounting the fibre. These results are similar to the unpublished observations briefly mentioned by Endo (1977). This alkaline pH-induced response was present even when the voltage-sensors had been inactivated by 1 min bathing in the Na^+ solution (see Lamb & Stephenson, 1990*a*) before exposure to the pH 8·0 (Na^+) solution (two fibres). The alkaline pH-induced response was completely blocked (along with the response to depolarization and 2 mM-caffeine) by 2 μM-Ruthenium Red; application of 30 mM-caffeine induced maximum force showing that the SR was still heavily loaded with Ca^{2+} (three fibres). Dettbarn & Palade (1991) report similar alkaline pH-induced Ca^{2+} release from SR vesicles.

Ca^{2+} uptake and leakage from the SR at different pH

At pH 7·1 in these skinned fibres, most of the Ca^{2+} released by a depolarization is retrieved by the SR rather than lost to the bathing solution (Fig. 1 in Lamb & Stephenson, 1990*a*). The small amount of Ca^{2+} lost to the bathing solution can be recovered in the period between successive depolarization. The depletion of SR Ca^{2+} during the bathing and depolarizations in both acidic and alkaline pH could be due to reduced retrieval of Ca^{2+}, particularly during each depolarization, and/or to 'leakage' of Ca^{2+} from the SR between depolarizations. This was examined further by bathing the fibres in the solution of different pH (50 μM-BAPTA) for 5 min without any depolarizations, then returning the fibre to the standard pH 7·1 solution (for 30 s) before depolarizing it (at pH 7·1). The response after 5 min in pH 6·1 was 95% (± 3%) of the control response in the same four fibres and for pH 7·6 was 34% (± 16%) of the control response in four other fibres. Bathing a fibre for 5 min in the standard pH 7·1 solution invariably increased the response to depolarization to above the control level, because of the small but appreciable Ca^{2+} loading in this solution over such a period of time. (Typically the response was between 105 and 120% of the control response, though of course this depends on how close to the maximum force the control response was in each case.) Thus, the reduced response seen in this experiment at pH 7·6 clearly implies that more pH leaked out during the 5 min period than was taken up by the SR. Similarly, the relatively unchanged response at pH 6·1 implies that the leak and uptake were nearly in balance. Nevertheless, this experiment only indicates the *net* difference between Ca^{2+} uptake and release and gives no indication of the absolute rates.

Ca^{2+} leakage from the SR

The leakage rate was assessed in isolation by preventing Ca^{2+} reuptake with solutions with 1 mM-free BAPTA to buffer the contaminating [Ca^{2+}] to very low levels (pCa > 8) and to rapidly chelate any released Ca^{2+}. After obtaining the normal depolarization-induced response in pH 7·1, the fibre was bathed for 5 min in the K^+

solution at a particular pH with 1 mM-BAPTA, returned to the standard K^+ solution (pH 7·1, 50 μM-BAPTA) for 45 s and depolarized (e.g. Fig. 4). The resulting response indicated how much releasable Ca^{2+} had been lost through leakage in the 5 min period. (From the values given earlier, it is clear that there was only a small amount

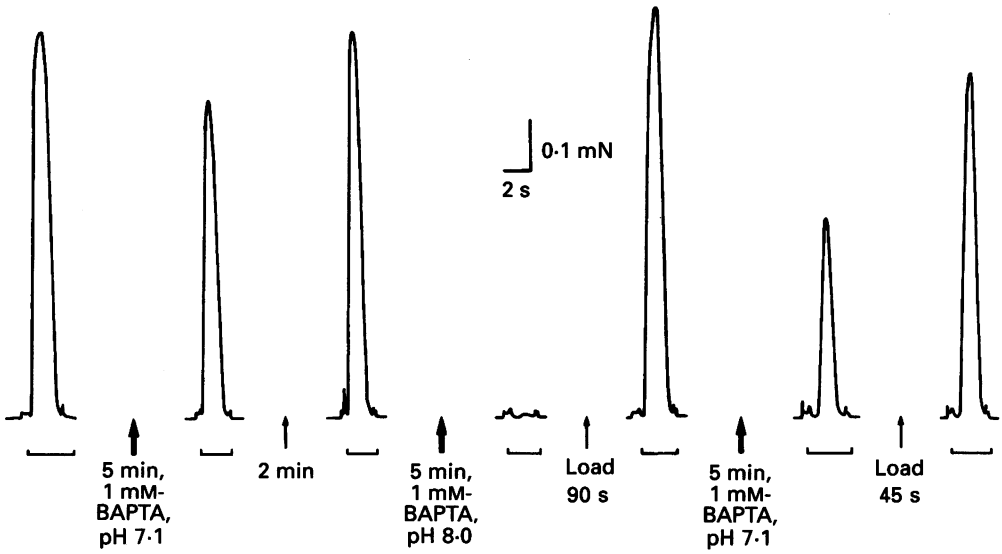

Fig. 4. Responses after 'leak' of Ca^{2+} from the SR at different pH. The fibre was bathed for 5 min (thick arrows) in a solution at a particular pH with very low [Ca^{2+}] (pCa > 8·0, 1 mM-BAPTA) to prevent any loading, then returned to the standard solution (pH 7·1, pCa 6·7, 50 μM-BAPTA) for 45 s to wash out the excess BAPTA and then depolarized by ChCl substitution (unlabelled bars). The size of the response indicated the amount of releasable Ca^{2+} remaining in the SR. Following depletion at each pH, the fibre was reloaded; when only slightly depleted the fibre was left in the standard solution (e.g. between second and third response) or, if it was substantially depleted, the fibre was bathed in the weakly buffered load solution (pCa 5·7, 50 μM-BAPTA; thin arrows) to accelerate the reloading. *L*, 2·5 mm; *D*, 33 μm).

of reloading during the 45 s equilibration period in the standard solution before depolarization.) The fibre was then reloaded with Ca^{2+} until the depolarization-induced response recovered to the control level, and the procedure was repeated at a different pH. The mean results are shown in Fig. 5. It was apparent that the response after the period in low [Ca^{2+}] was smaller if the procedure was repeated in the same fibre (e.g. compare the responses following the first and third exposures to low [Ca^{2+}] in Fig. 4). Consequently, the data is presented in two ways in Fig. 5: △ represent the mean of the responses at each pH obtained after the first exposure to low [Ca^{2+}] in fourteen fibres and ▲ represent the mean of the responses obtained using data from a first exposure at one pH and a second exposure at another pH. The former quantifies the responses without the complication of fibre deterioration, whereas the paired comparison in the latter (in which each pH was presented approximately an equal number of times in the first and second positions) shows that the trend apparent in the former is not due simply to fibre variation. It is clear from Fig. 5 that there is far more leakage of Ca^{2+} from the SR at alkaline pH than at

pH 7·1, and that the leak is, if anything, smaller at acidic pH. Thus, leakage of Ca^{2+} out of the SR between depolarizations can account for at least part of the progressive decrease in the depolarization-induced responses at alkaline pH.

However, it is clear that such leakage does not account for the SR depletion at acidic pH, which implies that at acidic pH the Ca^{2+} pump in the SR did not manage

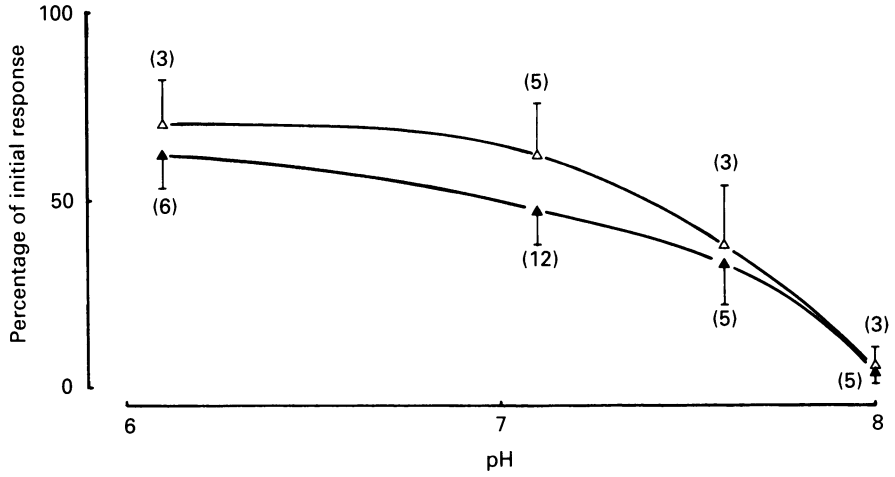

Fig. 5. Mean (±S.E.M.) depolarization-induced response, under standard conditions, following 5 min exposure to a low [Ca^{2+}] solution (pCa > 8·0, 1 mM-BAPTA) without stimulation (see Fig. 4). All values expressed as a percentage of the initial depolarization-induced response at pH 7·1. The reduction in the response gives an indication of the amount of releasable Ca^{2+} which 'leaked' out during the 5 min exposure. △ represents the responses obtained following the first exposure to low [Ca^{2+}] in each fibre. ▲ represents the average of the responses for each pH when each fibre was tested first at one pH and then at another (see text).

to recover most of the Ca^{2+} released by each depolarization before the Ca^{2+} diffused out of the preparation. This was confirmed by the following experiments on Ca^{2+} uptake.

Ca^{2+} uptake

Previous experiments with SR vesicles have shown that the activity of the SR pump is much lower at pH 6·0 than at pH 7·0, and actually slightly higher at pH 7·6 than at pH 7·0 (MacLennan, 1970; Shigekawa, Dougherty & Katz, 1978; Bishop & Al-Shawi, 1988). At pH 8·0, the activity of the pump is similar to that at pH 7·0, but is greatly depressed if free [Mg^{2+}] is greater than about 1 mM (Bishop & Al-Shawi, 1988). Fabiato & Fabiato (1978) examined Ca^{2+} reuptake in skinned skeletal muscle fibres by using caffeine to release Ca^{2+} from reloaded fibres and found that Ca^{2+} reuptake was reduced at acid pH (6·6 and 6·2) at low free [Ca^{2+}] (pCa 7·0) but not at high free [Ca^{2+}] (pCa 6·0). Ca^{2+} uptake by the SR at different pH was also examined here, in order (a) to measure the relative amount of Ca^{2+} taken up that can be released subsequently by depolarization and (b) to ensure that conditions were comparable with the depolarization-induced responses described above.

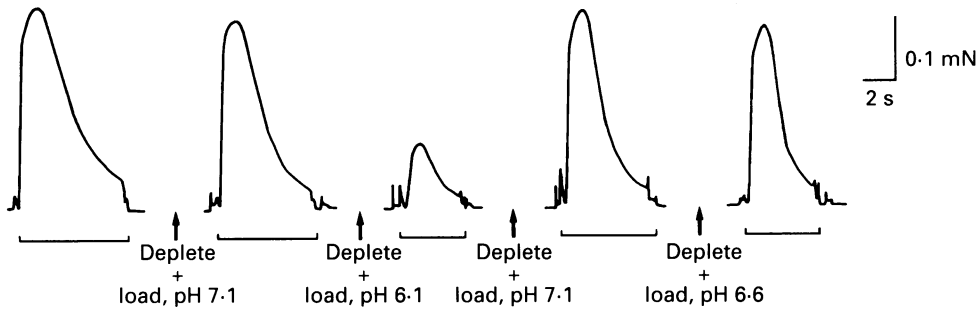

Fig. 6. Depolarization-induced responses after reloading with Ca^{2+} at different pH. The first response (extreme left) was elicited soon after mounting the fibre, without additional loading. The fibre was then depleted (by two depolarizations in 1 mM-BAPTA, pH 7·1 (not shown)), loaded in a K^+ solution with 1 mM-Ca-BAPTA (pCa 6·0) at a particular pH (arrows), washed twice (not shown: 0·5 mM-BAPTA, pH 7·1 for 30 s; 50 μM-BAPTA, pH 7·1 for 45 s) and then depolarized (by ChCl, indicated by unlabelled bars) under the standard conditions (pH 7·1, 50 μM-BAPTA). The resulting response is used as an indicator of the amount of Ca^{2+} reloaded at a given pH. *L*, 2·5 mm; *D*, 37 μm.

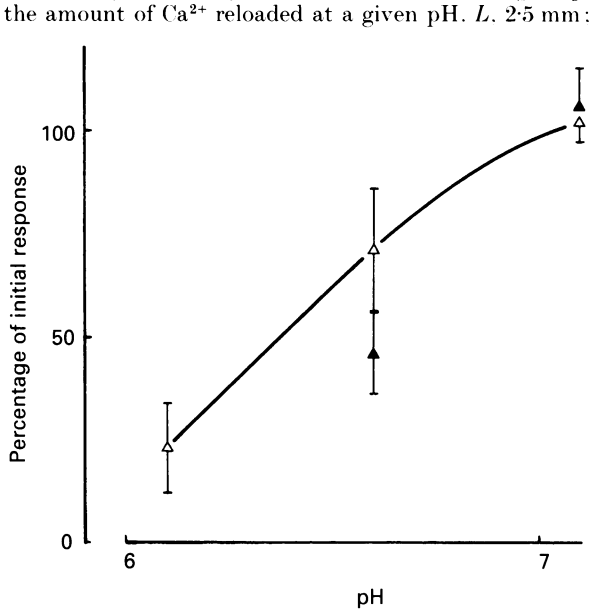

Fig. 7. Mean (±S.E.M.) depolarization-induced responses (in pH 7·1) after reloading a depleted fibre at various pH (see Fig. 6). Fibres were reloaded in a solution with 1 mM-Ca-BAPTA at pCa 6·0 (△) or pCa 7·0 (▲). All responses are expressed as a percentage of the initial depolarization-induced response (i.e. not additionally loaded) at pH 7·1. Data at pCa 7·0 were obtained at both pH 6·6 and 7·1 (in randomized order) in all six fibres, with 1 min loading in two fibres and 2 min in four fibres. Data at pCa 6·0 (30 s load) were obtained for all three pH values (in randomized order) in three fibres and only at two pH values (pH 6·1 and 7·1) in another fibre.

Fibres were first depleted by two depolarizations in solutions at pH 7·1 with 1 mM-BAPTA (pCa > 8·0). No depolarization-induced response could be elicited in any fibre after such a procedure without reloading the SR with Ca^{2+}, and the greatly reduced response to application of 30 mM-caffeine also showed that the SR Ca^{2+} was

considerably depleted (see also Fig. 2 in Lamb & Stephenson, 1990*b* and Fig. 8 in Lamb & Stephenson, 1991). The fibres were then reloaded in solutions at normal or acidic pH, in which the free [Ca^{2+}] within the fibre should have been reasonably well buffered; all solutions had 1 mM-Ca-BAPTA and appropriate amounts of BAPTA to produce a low (pCa 7·0) or relatively high (pCa 6·0) free [Ca^{2+}] (see Methods). (Loading in alkaline conditions was not studied because the higher leak of Ca^{2+} (see above) would have confounded the interpretation of the results.) After 30 s loading in pCa 6·0 (e.g. Fig. 6) or 1 or 2 min loading in pCa 7·0, each fibre was rinsed for 30 s in a low [Ca^{2+}] solution (0·5 mM-BAPTA with no added Ca^{2+}), then rinsed again for 45 s in the standard K^+ solution (pH 7·1) and depolarized. The resulting responses indicated that Ca^{2+} uptake was substantially reduced at acidic pH, at both pCa 6·0 and 7·0 (Figs 6 and 7). This readily explains why the SR lost substantial amounts of Ca^{2+} during depolarizations at acidic pH.

DISCUSSION

Depolarization-induced responses at various pH

The results presented here show that depolarization can elicit large force responses at any pH between 6·1 and 8·0, providing that the SR has not already been depleted of Ca^{2+} (Figs 1–3). Thus, despite the non-linear relationship between [Ca^{2+}] and force, it is clear that depolarization could trigger massive release of Ca^{2+} at every pH examined, even at very acid pH. This is consistent with previous studies with intact fibres which showed that acidifying the myoplasm by application of CO_2, only reduced the tetanic force by a small amount (15–30 %), apparently by predominantly affecting the contractile apparatus (see references in Introduction). Thus, the almost complete block of Ca^{2+}-activated opening of isolated Ca^{2+} release channels at pH 6·5 (Ma *et al.* 1988; Rousseau & Pinkos, 1990) does not indicate the true effect of intracellular pH on E–C coupling. The findings presented here again emphasize that normal E–C coupling does not purely involve a simple Ca^{2+}-induced Ca^{2+}-release mechanism (Endo, 1985; Lamb & Stephenson, 1990*b*, 1991).

At both acidic and alkaline pH, there was a decline in the response to successive depolarizations (Fig. 3). At acidic pH, this progressive decline appears to be entirely due to depletion of releasable Ca^{2+} in the SR. Although the leak of Ca^{2+} from the SR is similar to or even smaller than at pH 7·1 (Fig. 5), the Ca^{2+} pump is much less able to retrieve the Ca^{2+} released by depolarizations, both when the 'myoplasmic' [Ca^{2+}] is elevated (pCa 6·0) or close to resting levels (pCa 7·0) (Figs 6 and 7). Consequently, more Ca^{2+} is lost to the bathing solution during a depolarization than can be taken up during the 1 min interval between successive depolarizations. The fact that the pump cannot lower the 'myoplasmic' [Ca^{2+}] as rapidly at acidic pH, also readily explains the longer duration of the depolarization-induced response under those conditions, particularly when it is remembered that the affinity of the contractile apparatus for Ca^{2+} is lower at acidic pH. Thus, the prolonged response at acidic pH in these experiments can be explained in a similar manner to the effect of elevated CO_2 in slowing the relaxation of intact fibres after a long tetanus (Curtin, 1986).

At alkaline pH there are at least two other factors contributing to the decline of the responses to successive depolarizations. Firstly, at alkaline pH, a considerable

amount of Ca^{2+} 'leaks' out from the SR, presumably continuously, both at normal $[Ca^{2+}]$ (see text) and at low $[Ca^{2+}]$ (e.g. Fig. 4). This can substantially deplete the SR within 5 min. Secondly, taking into account that the contractile apparatus is actually more sensitive to Ca^{2+} at higher pH, the fact that the response 30 s after returning to pH 7·1 was considerably larger than the preceding response at alkaline pH (see text and Fig. 2), implies that depolarization was less able to release Ca^{2+} at alkaline pH than at pH 7·1. This phenomenon might result from the increased amount of Ca^{2+} bound by calsequestrin in the SR at alkaline pH (about 10% more at pH 8·0, MacLennan & Wong, 1971). Alternatively, the drop in $[H^+]$ (i.e. rise in pH) might reduce the size of a counter current (probably carried by K^+, Mg^{2+} and the highly mobile H^+) which is probably necessary to balance the current produced by Ca^{2+} efflux from the SR (Somlyo, Gonzalez-Serratos, Shuman, McClennan & Somlyo, 1981). The longer duration of the response at alkaline pH can be explained by the increased sensitivity of the contractile apparatus to Ca^{2+} and by an increase in the open-time of the Ca^{2+}-release channels (see next section).

Ca^{2+} leakage and the pH dependence of Ca^{2+} channel opening

It seems likely that the leakage of Ca^{2+} at alkaline pH (text and Figs 4 and 5) and the alkaline pH-induced response in heavily loaded fibres (Fig. 2) are the result of the same underlying phenomenon, namely an increase in the mean open time of the Ca^{2+} release channels at alkaline pH. Thus, the results presented here suggest a pH dependence of Ca^{2+} channel opening similar to that described in the single-channel experiments of Ma *et al.* (1988) and Rousseau & Pinkos (1990) for a single, relatively high $[Ca^{2+}]$ (2 or 10 μM) in the absence of Mg^{2+} and ATP. The Ca^{2+} leakage results presented here imply that the release channels do not 'spontaneously' open very frequently in this preparation with physiological [ATP] and $[Mg^{2+}]$ and a functioning voltage sensor, because otherwise the SR would have been depleted of Ca^{2+} within milliseconds to seconds. Nevertheless, the results do imply that the mean open time, although very low, does increase with pH. Thus, given that the mean open time increases with higher $[Ca^{2+}]$ (Ma *et al.* 1988), it seems reasonable to assume that the leakage can increase to such an extent in heavily loaded fibres at pH 8·0, that the release becomes self-reinforcing, thereby producing the large alkaline pH-induced responses. This would be consistent with the findings that such release was unaffected by inactivation of the voltage sensors but was completely suppressed by 2 μM-Ruthenium Red, a well-known blocker of the calcium release channel (Ma *et al.* 1988). However, it should be emphasized that this uncontrolled, self-reinforcing response does not play the major role in normal E–C coupling, where Ca^{2+} release stays under the control of the voltage sensors (Lamb & Stephenson, 1990*b*, 1991, and references therein).

pH and fatigue

The results reported here show that decreasing the pH to 6·1 has little if any effect on the depolarization-induced release of Ca^{2+}, which as mentioned above is consistent with reports of acidification of intact fibres with CO_2. Thus, the drop in pH observed in fatigued muscle probably exerts its major effect on the tetanic response by reducing the maximum force of the contractile proteins to a small extent, as

suggested by Lännergren & Westerblad (1991). Thus, the eventual decline in Ca^{2+} release from the SR in fatigued fibres (Allen *et al.* 1989; Lee, Westerblad & Allen, 1991) is not accounted for by the decline in pH (approximately from pH 7·0 to 6·5, Renaud *et al.* 1986). It is possible that the decline in Ca^{2+} release is due in part to a rise in free $[Mg^{2+}]$ following the hydrolysis of ATP to ADP, as high $[MG^{2+}]$ has been shown recently to decrease depolarization-induced release (Lamb & Stephenson, 1991). The displacement of bound Mg^{2+} by the released Ca^{2+}, could further contribute to this rise in free $[Mg^{2+}]$. A further factor contributing to the decline in Ca^{2+} release during fatigue could be increased binding of Ca^{2+} within the SR by inorganic phosphate. Finally, the higher 'resting' $[Ca^{2+}]$ in fatigued fibres (Allen *et al.* 1989; Lee *et al.* 1991) is quite possibly the result of the intracellular acidosis. Such a drop in pH only slightly reduces the leakage of Ca^{2+} out of the SR (Fig. 5), whereas it considerably reduces Ca^{2+} uptake (Fig. 7). Consequently, assuming that there is relatively little Ca^{2+} movement across the sarcolemma, the 'resting' $[Ca^{2+}]$ in a fatigued fibre (where of course efflux of Ca^{2+} must equal re-uptake) will be higher than in an unfatigued fibre at pH 7·1.

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